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Vaccination with post-translational modified, homocitrullinated peptides induces CD8 T-cell responses that mediate antitumor immunity

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ABSTRACT

Background Post-translational modification of proteins has the potential to alter the ability of T cells to recognize major histocompatibility complex (MHC) class -I and class-II restricted antigens, thereby resulting in altered immune responses. One such modification is carbamylation (homocitrullination) that results in the formation of homocitrulline (Hcit) residues in a non-enzymatic reaction of cyanate with the lysine residues in the polypeptide chain. Homocitrullination occurs in the tumor microenvironment and CD4-mediated immune responses to Hcit epitopes can target stressed tumor cells and provide a potent antitumor response in mouse models.

Methods Homocitrullinated peptides were identified and assessed in vitro for HLA-A2 binding and in vivo in human leukocyte antigen (HLA) transgenic mouse models for immunogenicity. CD8 responses were assessed in vitro for cytotoxicity and in vivo tumor therapy. Human tumor samples were analyzed by targeted mass spectrometry for presence of homocitrullinated peptides.

Results Homocitrullinated peptides from aldolase and cytokeratin were identified, that stimulated CD8-mediated responses in vivo. Modified peptides showed enhanced binding to HLA-A2 compared with the native sequences and immunization of HLA-A2 transgenic mice generated high avidity modification specific CD8 responses that killed peptide expressing target cells. Importantly, in vivo the homocitrullinated aldolase specific response was associated with efficient CD8 dependent antitumor therapy of the aggressive murine B16 tumor model indicating that this epitope is naturally presented in the tumor. In addition, the homocitrullinated aldolase epitope was also detected in human tumor samples.

Conclusion This is the first evidence that homocitrullinated peptides can be processed and presented via MHC-I and targeted for tumor therapy. Thus, Hcit-specific CD8 T-cell responses have potential in the development of future anticancer therapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Post-translational modifications of proteins provide targets for T cells that have not been subject to central tolerance. Homocitrullination of proteins occurs in tumors and can be targeted by CD4 T-cell responses.

WHAT THIS STUDY ADDS

⇒ This study provides the first evidence that homocitrullinated proteins in tumors can also be targets for CD8 T-cell responses.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This research suggests that post-translational modification of proteins provide efficient targets in tumors for both CD4 and CD8 T-cell-mediated therapies.

INTRODUCTION

Post-translational modifications (PTMs) are important parts of normal physiology that result in the alteration of specific amino acids either in a spontaneous process or through controlled enzymatic reactions. In addition to controlling the structural properties of proteins, these PTMs alter the antigenicity of self-proteins and can result in altered processing and presentation of new epitopes by major histocompatibility complex (MHC) molecules.[1] Typically, the epitopes presented to CD8 T cells are derived from cytosolic proteins that undergo proteasomal degradation and loading onto MHC-I molecules.[2] Binding of peptide to MHC-I involves the formation of conserved hydrogen bonding between the N and C termini of peptide and residues within the MHC heavy chain. These interactions play crucial roles in limiting the length of MHC-I presented peptides to primarily 8-10aa.[3] However, peptides between 11aa to 16aa in length have been reported that form a bulging conformation. The extra residues between the primary anchor residues bulge outward from the peptide binding cleft to maintain the conserved hydrogen bonding

interactions between MHC class I binding site and the peptide.[4 5] In contrast to MHC-I molecule, MHC-II molecules on antigen presenting cells typically present peptide derived from exogenous antigen to CD4 T cells.[6] Due to having an open peptide binding groove, MHC-II molecules can accommodate larger peptides of 13–25 residues.[7] Citrullinated and homocitrullinated epitopes are known to induce efficient CD4 T-cell mediated immunity in vivo.[8–13] Homocitrullination occurs in a process called carbamylation that results in the conversion of lysine residue to homocitrulline (Hcit) when isocyanic acid reacts with the ε-NH2 group on the side chain of lysine. Isocyanic acid is formed by the action of myeloperoxidase (MPO) on thiocyanate.[14 15] Under chronic inflammatory conditions, MPO can be released from immune cells such as neutrophils, monocytes, and certain tissue macrophages.[16] It has previously been shown that myeloid-derived suppressor cells (MDSCs) can be a source of MPO in the tumor microenvironment that can drive homocitrullination.[17 18] Several PTMs such as citrullination and homocitrullination have been implicated in the etiology of autoimmune diseases,[19] particularly as the targets of antibody responses.

In this study, we have characterized two Hcit peptides, one from aldolase A (Aldo) and one from cytokeratin 8 (Cyk8) as targets for CD8 T-cell-mediated anticancer therapy. Aldo is a glycolytic enzyme, the expression of which has been reported to be upregulated in several cancers.[20] Cyk8 belongs to a family of intermediate filament proteins that are important for the cytoskeleton of epithelial cells. High expression of Cyk8 is a prognostic and diagnostic marker of a number of epithelial cell cancers.[21–24] We have demonstrated that the Hcit modification enhances peptide binding to HLA-A2 and immunization with modified peptides induces strong Hcit specific CD8 responses in HLA-A2 transgenic mice. These Hcit specific CD8 responses are capable of in vivo tumor rejection when Hcit peptides are naturally presented by tumors.

MATERIAL AND METHODS

Unless otherwise stated reagents were obtained from Sigma-Aldrich and antibodies from Thermo Fisher.

Peptides

Peptides were selected for potential A2 binding using the Immune Epitope Database (IEDB) (http://www.iedb.org/) binding predictions[25] using wild type (wt) sequence. Peptides (online supplemental table 1) containing lysine or Hcit were synthesized at >90% purity (GenScript or Alta Bioscience), stored lyophilized at −80°C and then reconstituted at 1 mg/mL in 20% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS).

Animals and cell lines

HLA-A2 (HHDII/murine I-Ab Pasteur Institute) or HLA-A2/DR1 (HHDII/DR1, Pasteur Institute) transgenic mice aged 8–12 weeks were used.

The murine melanoma B16F1 cell line (ATCC-CRL-6323) obtained from American Type Culture Collection was engineered with mouse MHC-I knocked out and transfected with HHDII (transgenic HLA-A2) using plasmids as previously described but retains expression of murine MHC-II.[26] Cells were cultured in RPMI (Roswell Park Memorial Institute) medium 1640 with L-glutamine (2 mM), 10% fetal calf serum (FCS) and appropriate antibiotics to maintain plasmids. Parental T2 cell line (ATCC-CRL-1991) or T2 cell line transfected with HLA-DR1 was used for peptide pulsing experiments. Cell lines used were mycoplasma-free, authenticated by suppliers (short tandem repeat profiling), and used within 10 passages.

Immunization protocol

Synthetic lyophilized peptides (GenScript or Alta Bioscience) were reconstituted at 1 mg/mL in 20% DMSO in PBS immediately prior to injection. Transgenic mice were subcutaneously injected with 25 µg of each peptide either individually or as a pool with 5 µg each of CpG ODN 1826 and monophosphoryl lipid A (Invivogen) made up to 100 µl in PBS. Immunizations were performed on days 1, 8 and 15. Spleens were harvested on day 21 for immune response analysis. Groups of n=3 selected through statistical power calculation were used on independent occasions.

For in vivo antitumor response, HHDII mice were implanted subcutaneously with 5×10^4 B16F10 HHDII cells per mouse on day 1. Immunization with peptide and adjuvant mixture as described above, was performed on days 4, 8 and 11. In relevant studies, anti-mouse CD4 (Bio X Cell, GK1.5, BE003) or anti-mouse CD8 (Bio X Cell, 2.43, BE0061) antibodies were given intraperitoneally at 500 µg on day 4, and 300 µg on days 8 and 11. Mice were checked twice weekly for tumor growth and were euthanized humanely once tumors approached or about to approach 15 mm in diameter. Groups of n=5–10 selected through statistical power calculation were used on independent occasions.

In vitro splenocyte culture

Splenocytes were pooled within each group and plated out into 24-well plates at 5×10^6 cells per well in culture media (RPMI medium 1640 (GIBCO/BRL) supplemented with 10% FCS (Sigma), 2 mM L-glutamine (Sigma) and sodium bicarbonate buffered with additional 20 mM HEPES (Sigma), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 50 µM 2-mercaptoethanol (Thermo Fisher) and 20 IU anti-mouse interleukin-2 (IL-2, Miltenyi Biotec, 130-120-331, 1.4×10^6 IU/mL). Cells were stimulated with between 0.1 and 10 µg/mL of peptide. Cells were then incubated at 37°C until further use. On day 5/6, cells were harvested and subjected to histopaque (Histopaque, –1083, SIGMA, 10831–100 ML) isolation to remove dead cell debris. After isolation, viable lymphocytes were counted and plated again in a 24-well plate as ~2×10^6 cells per well and rested until further use.

ELISpot assays

Interferon γ (IFNγ) enzyme-linked immunosorbent spot (ELISpot) assay was performed using murine ELISpot kits (Mabtech) according to manufacturer's instructions. Briefly, 5×10^5 splenocytes were seeded into quadruplicate wells of 96-well Immobilin P-plate coated with capture antibody (10 µg/mL). Synthetic peptides diluted in 20% DMSO in PBS were used at 10 µg/mL (unless otherwise stated) in relevant wells with or without 20 µg/mL anti-mouse CD4 (Bio X Cell, clone GK1.5) or anti-mouse CD8 (Bio X Cell, clone 2.43) for blocking studies. Unless stated otherwise, LPS (Sigma, L2387) was used at 5 µg/mL as a positive control as a way to assess splenocyte viability. For avidity measurement, peptides were serially diluted 10-fold starting from 10 µg/mL to as low as 1×10^{-6} µg/mL. Following 40 hours incubation at 37°C in 5% CO_2, biotinylated anti-IFNγ antibody along with streptavidin alkaline phosphatase and chromogenic substrate were used to detect IFNγ response. Spots were counted using an automated plate reader (Cellular Technologies). For ELISpot assay from cultured splenocytes, 1×10^5 cells were used per well and the assay was developed after 20 hours.

Flow cytometric staining of animal tissue and cells

Cells were first stained with LIVE/DEAD stain (Thermo Fisher, L34967) for 10 mins and washed prior to incubation with Fc gamma receptor blocking reagent (Miltenyi Biotec, 130-092-575) for another 10 mins. Cells were stained with extracellular antibodies, fixed and permeabilized using fixation and permeabilization buffers (Thermo Fisher) followed by staining for intracellular markers and appropriate secondary antibodies where required. Stained samples were fixed and analyzed using MACSQuant16 flow cytometer and MACSQuantify software V.2.13.3. The details of the antibodies used are presented in online supplemental table 2.

T2 binding assay

T2 cells were washed in RPMI medium and adjusted to 2×10^6 cells/mL. Peptides were serially diluted in RPMI at concentrations of 400 µg/mL, 40 µg/mL and 4 µg/mL. Recombinant human β_2-micoglobulin (β_2M) (BD Biosciences, 551089) was also prepared at 800 ng/mL. In 96-well U bottom plate, 100 µL of cells were incubated with 50 µL of peptide and 50 µL of β_2M and incubated at 37°C for overnight, followed by addition of brefeldin A (BD Biosciences) for 1 hour prior to washing and staining with anti-human HLA-A2 (online supplemental table 2). Analysis was performed on the MACSQuant16 flow cytometer. Median fluorescence intensity (MFI) of the HLA-A2 fluorescein isothiocyanate (FITC) antibody staining was used to determine the strength of peptide binding to MHC-I.

Cytotoxic killing assay

The assay was performed using CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega, G1780) according to manufacturer's instructions. In brief, target cells were pulsed with 10 µg/mL of the relevant peptide for 2 hours at 37°C in serum-free medium, washed and resuspended in assay medium (RPMI without phenol red (Thermo Fisher, 32404014), 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin- 0.1 mg/mLstreptomycin) at 2×10^5 cells/mL. Effector cells were harvested, washed and resuspended in the assay medium to provide specific effector to target ratios. The assay was set up in 96-well round bottom plate in a final volume of 100 µL/well and plates were centrifuged at 250 g for 4 mins to ensure maximum effector and target cell contact. Following 6 hours incubation at 37°C, the plate was centrifuged at 250 g for 4 mins. Then, 50 µL of supernatant per well was transferred to a 96-well flat bottom tissue culture plate and 50 µL of substrate mix was added, incubated at room temperature and the reaction was stopped with 50 µL of stop solution. Absorbance was recorded at 490 nm.

Tumor lysate preparation and mass spectrometry analysis

Lysates made from frozen human tumor samples (Tissue Solutions, online supplemental table 3) in 4M Gua*HCl 100 mM Tetraethylammonium bromide (TEAB) were assessed for protein concentration using modified Bradford assay; 4 mg each were labeled with TMTpro zero reagent, and then spiked with heavy TMTpro-labeled peptide standard mix prepared from synthetic versions of the peptides of interest to 90pM per 4 mg. All samples were filtered through ultrafiltration cartridges (50 kDa cut-off, Amicon) to obtain the flow-through peptidomics samples. The flow-throughs were reduced (1 mM TCEP), alkylated (7.5 mM iodoacetamide), hydroxylamine-treated, and purified via HLB (Oasis Vac RC 30 mg cartridges). The resulting eluates were fractionated using the Pierce High pH Reversed-Phase Peptide Fractionation Kit according to manufacturer's instruction for TMT-labeled peptides to initially produce eight fractions. Fractions 1–6 were combined and fractions 7 and 8 used as such, to finally produce three fractions.

Samples were analyzed using an Orbitrap Fusion Tribrid Mass Spectrometer, operated in conjunction with an Easy nLC 1200 (both Thermo Fisher Scientific). The chromatographic system consisted of a 2 cm 75 um ID PepMap trap column and a 55 cm 75 uM ID EasySpray analytical column with integrated emitter. From the thermostatted autosampler, samples were loaded onto the trap column at a pressure controlled maximum flow rate in 100% aqueous solvent (0.1% formic acid in water). The final gradient for optimal separation of the target peptides started at 20% organic solvent (80% acetonitrile, 0.1% formic acid) and increased to 43% organic solvent over 60 min, followed by a wash-out phase at 100% organic phase.

The mass spectrometer was operated in targeted mode, with interspersed full scans every 3 s. Full scans were acquired at 240K resolution, with a maximum fill time of 100 ms and a normalized Automatic gain control (AGC) target of 250%. The precursor isolation list consisted of the preferred charge state for each target peptide in light

and heavy variants. If a peptide contained methionine, both the oxidized and non-oxidized form were monitored (unless the non-modified form was highly abundant). Precursor isolations were scheduled within 5–8 min retention time windows based on a control sample run immediately before the sample sequence. MS2 scans were acquired at 50K resolution with 86 ms maximum injection time and a normalized AGC target of 1000%. Peptides were fragmented by higher energy collision dissociation at a normalized collision energy of 30.

Data files were loaded into Skyline (University of Washington) and peak boundaries were manually set based on the signal in the internal standard channel. Retention time criteria and precursor mass accuracy were used when precursors were too close to each other to be differentiated by the peak extraction algorithm. For the qualitative analyses, all peaks were inspected manually.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software V.9.3.1. Comparative analysis of the ELISpot results was performed by applying paired or unpaired analysis of variance or Student's t-test as appropriate with p values calculated accordingly. Comparison of tumor survival was assessed by log-rank (Mantel-Cox) test. P<0.05 values were considered statistically significant.

RESULTS

Post translationally modified homocitrullinated peptides can stimulate CD8-mediated immune responses

We have previously used long peptides to identify homocitrullinated and citrullinated peptides that induce CD4-mediated responses.[8 17] Longer peptides can also encode nested CD8 epitopes and can stimulate both CD4 and CD8 T-cell-mediated responses.[27] While screening for long peptides from Aldo and Cyk8 proteins, we sought to determine if any potential CD8 epitopes were included. Using IEDB binding prediction software, we identified peptides predicted to bind to the HLA allele HLA-A*0201 (online supplemental table 1). Peptides were selected to contain lysine residues in the predicted core MHC binding regions. However, prediction software does not take into account the effect of the modification on the peptide structure and MHC binding so peptides with a range of binding scores were included.

HLA-A2 (HHDII/DR1) transgenic mice were immunized (figure 1A, immunization regime) with long peptides from Aldo and Cyk8 in which lysine was replaced with homocitrulline. These transgenic mice express HHDII which is a chimeric MHC-I molecule with the alpha 3 domain of murine MHC-I and the peptide binding groove and the alpha 1 and alpha 2 domains of human HLA-A2 (HLA-A*0201) alongside the human HLA-DR1 molecule and have been knockout for any mouse MHC-I or II genes.[28] Strong responses to the Aldo 204-219Hcit (p=0.0071), Cyk8 112-132Hcit (0.0144) and Cyk8 371-388Hcit (p=0.0068) peptides, but not to Aldo 74-93Hcit were observed (figure 1B). Response to Aldo 140-157Hcit could also be seen but it did not reach significance (p=0.1988). The use of long peptides meant there was potential for these peptides to stimulate both CD4 and CD8 responses. To confirm if responses were CD8 or CD4 mediated, responses in mice immunized with the long Hcit peptide were assessed in the presence of CD4 or CD8 blocking antibody. Although it did not reach statistical significance, it is evident from representative ELISpot images (online supplemental figure 1) that Aldo 140-157Hcit and Cyk8 371-388Hcit responses were blocked in the presence of the CD4 blocking antibody but not in the presence of the CD8 blocking antibody confirming the presence of CD4-mediated responses to these peptides that is consistent with previous work (figure 1C).[17] Addition of either anti-CD8 or anti-CD4 antibody significantly reduced the immune response induced by Cyk8 112-132Hcit peptide suggesting the presence of both CD4 and CD8 epitopes within this sequence (figure 1C). However, the strong response to Aldo 204-219Hcit was not affected by either of the blocking antibody (figure 1C). To further investigate the presence of CD4 or CD8 epitopes, splenocytes were cultured in the presence of peptide to expand peptide specific T-cell populations and the response to peptide restimulation was assessed in the presence of the CD4 or CD8 blocking antibody. Responses to both Aldo 204-219Hcit and Cyk8 112-132Hcit were significantly blocked by CD8 blocking antibody providing evidence for nested CD8 epitopes in both peptides, whereas blocking with the CD4 antibody had no effect (figure 1D). This data provides evidence that homocitrullinated peptides can stimulate CD8 T-cell responses in addition to CD4 T-cell responses.

CD8 responses to homocitrullinated peptides are modification specific and can be mapped to shorter sequences

CD4 T-cell responses have been described to specifically recognize the Hcit modification of lysine,[17 29] however it is not known if CD8 T cells are also able to specifically recognize this PTM. Hcit peptide stimulated responses were therefore also tested for reactivity to the wt peptides in HLA-A2 (HHDII/DR1 and HHDII, the latter expresses human MHC-I and mouse MHC-II I-Ab) transgenic mouse models. Both the Aldo 204-219Hcit and Cyk8 112-132Hcit peptides stimulated strong modification specific responses that were significantly better than the response to equivalent wt peptides (figure 2A and online supplemental figure 2). Representative ELISpot images are shown in online supplemental figure 1. Since both peptide sequences are homologous in mice and humans, it is likely that T cells specific to these epitopes are subjected to similar tolerance mechanisms in both species. Hence, it can be suggested that modified self-peptide specific CD8 T cells can escape central tolerance. To determine if a T-cell repertoire exists to the wt sequence, immune responses were assessed in mice immunized with the wt peptides. HHDII/DR1 mice immunized with the Cyk8 112–132wt and Aldo 204–219wt peptides

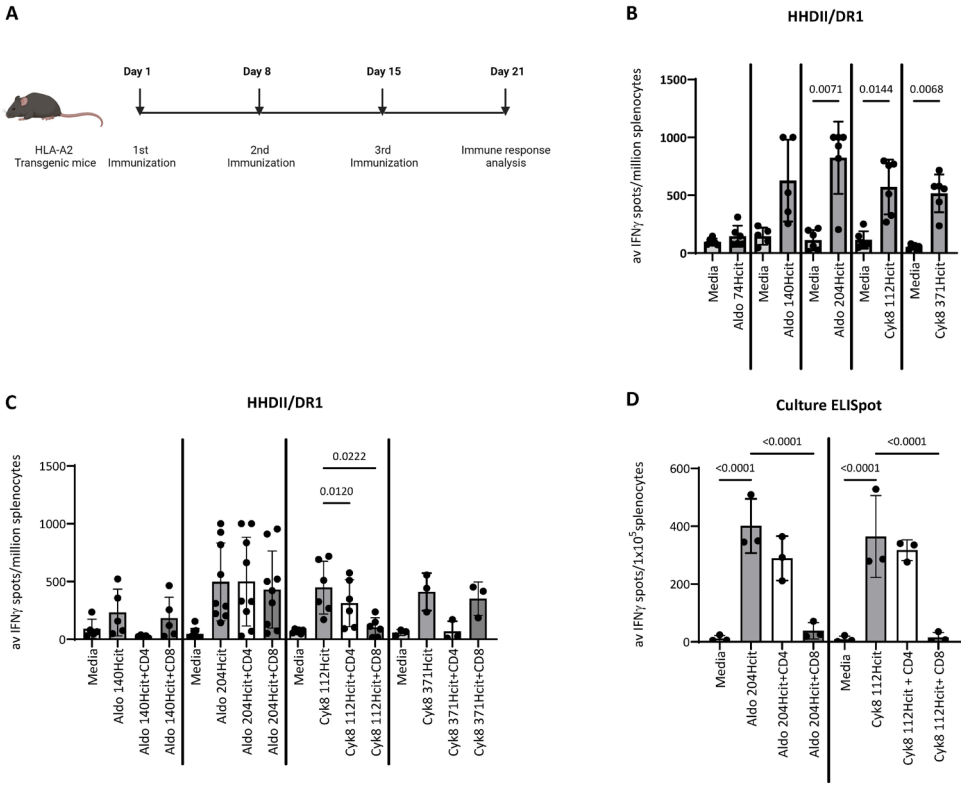

Figure 1 Long homocitrullinated peptides stimulate CD8-mediated immune responses in HLA-A2 transgenic mice. (A) Immunization schedule. Immune responses in HLA-A2 transgenic (HHDII/DR1) mice immunized with individual peptides containing Hcit residues (B) were assessed by IFNγ ELISpot assay. Mice were immunized on days 1, 8 and 15 and splenocytes analyzed on day 21. Splenocytes from individual HHDII/DR1 mice immunized with Aldo 140-157Hcit, Aldo 204-219Hcit, Cyk8 112-132Hcit or Cy8 371-388Hcit, were subjected to peptide restimulation alone or in combination with CD4 or CD8 blocking ex vivo (C). Symbols represent mean IFNγ spots/million splenocytes for individual mouse (B and C). Aldo 204-219Hcit and Cy8 112-132Hcit responses (pooled splenocytes within each group where n=3) were also assessed with or without CD4 or CD8 antibody after 7-day in vitro culture (D). Peptide specific responses were assessed by IFNγ ELISpot assay. Data are collated from or representative of independent studies where n=3. Significant p values (p<0.05) are shown where statistical analysis was performed using paired (B and C) or unpaired (B and D) ANOVA. Aldo, aldolase A; ANOVA, analysis of variance; AV, average;Cyk8, cytokeratin 8; ELISpot, enzyme linked immunosorbent spot; Hcit, homocitrulline; HLA, human leukocyte antigen; IFNγ, interferon γ.

failed to stimulate detectable IFNγ immune responses to either the wt or Hcit peptides suggesting that a T-cell repertoire may not exist to these wt peptides (figure 2B). Taken together these data suggest that Cyk8 112-132Hcit and Aldo 204-219Hcit peptides can induce Hcit specific immune responses that are mediated by CD8 T cells.

We subsequently sought to identify the shorter CD8 epitopes by screening IEDB predicted HLA-A2 epitopes (online supplemental table 1) in HLA-A2 transgenic (HHDII/DR1) mice. Mice immunized with Aldo 204-219Hcit showed significant responses to the immunizing peptide as well as strong responses to the shorter Aldo 209-217Hcit peptide (figure 2C). Similarly, Cyk8 112-132Hcit peptide immunized mice showed significant responses to the longer peptide and also to the shorter Cyk8 117-125Hcit peptide (figure 2C). Immune responses to all the other shorter peptides selected based on IEDB binding prediction were minimal (online supplemental figure 3).

To induce an effective antitumor response, the functional avidity of the CD8 response is important.[26 30 31] We determined the avidity of the CD8 T cells to the Aldo 204-219Hcit, Aldo 209-217Hcit and Cyk8 112-132Hcit, Cyk8 117-125Hcit peptides in HHDII/DR1 mice immunized with either Aldo 204-219Hcit or Cyk8 112-132Hcit. Higher avidity responses to the shorter peptides were seen compared with the longer ones. The EC_{50} (half maximal effective concentration) of the shorter Aldo 209-217Hcit peptide response was $1.796e^{-010}$, compared with $2.668e^{-007}$ for the longer Aldo 204-219Hcit peptide (figure 2Di). The EC_{50} of the shorter Cyk8 117-125Hcit peptide response ($7.817e^{-011}$) also appeared to be lower compared with that of longer Cyk8 112-132Hcit ($7.083e^{-007}$) (figure 2Dii). This is most likely due to better binding of the short peptides to MHC-I than the longer peptides.[3] Implying that Aldo209-217Hcit and Cyk8 117-125Hcit peptides are the most optimal for MHC-I binding and the longer peptides are likely to be processed to these short sequences for presentation in vivo. This data shows that it is possible to identify the optimal MHC-I binding epitopes. These epitopes correlate well with peptides predicted to bind well to HLA-A2 (online supplemental table 1).

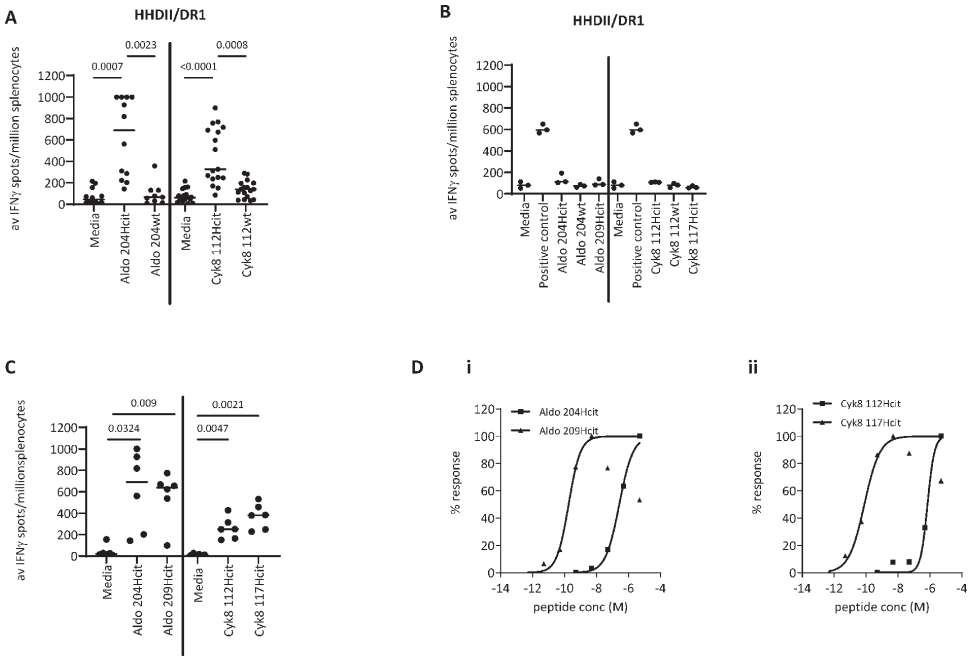

Figure 2 Homocitrullinated peptide immunization can induce modification specific CD8 responses. IFNγ ELISpot responses were examined in HLA-A2 transgenic (HHDII/DR1) mice on day 21 after three immunizations (days 1, 8 and 15) with Aldo 204-219Hcit or Cyk8 112-132Hcit peptide (A, C and D). Immune responses to vaccination with Aldo 204–219 wt or Cyk8 112–132 wt peptide were assessed in HHDII/DR1 mice (B). IFNγ responses were tested against the long Hcit and native (wt) sequences (A and B) and short Hcit 9aa CD8 peptides (B and C). LPS was added as positive control to confirm splenocyte viability (B). Symbols represent mean IFNγ spots/million splenocytes for individual mouse and the line represents median value between mice. Significant p values are shown from paired ANOVA analysis. Titrations of the short and long Aldo Hcit (Di) or Cyk8 Hcit (Dii) peptides were performed to determine the avidity of both the longer and shorter peptides (in HHDII/DR1 mice). For each concentration of peptide, data from three individual mice were collated and normalized avidity graphs are shown. Aldo, aldolase A; ANOVA, analysis of variance; AV, average; Cyk8, cytokeratin 8; Hcit, homocitrulline; IFNγ, interferon γ; wt, wild type.

Homocitrulline modification can enhance peptide binding to HLA-A2

To determine if responses reflect peptide binding affinity, the binding affinity of the longer and all the shorter Hcit peptides were assessed in a T2 binding assay. T2 is a transporter-associated with antigen processing (TAP) deficient cell line that expresses low levels of HLA-A2 on the cell surface. Due to the TAP deficiency, these cells are incapable of processing antigens and can only present exogenous peptides on MHC-I. Binding of peptides to HLA-A2 stabilizes the complex leading to an increase in expression that can be detected by fluorescent labeled HLA-A2 antibody. Data from the T2 binding assay demonstrated minimal binding of the longer Hcit peptides which was expected as long peptides rarely bind strongly to MHC-I[32] (figure 3A,B). Among all the shorter peptides selected, only Aldo 209-217Hcit and Cyk8 117-125Hcit peptides showed enhanced binding to HLA-A2 which correlated with the immunogenicity data (figure 3A,B). Further comparison of the Aldo 209-217Hcit and Cyk8 117-125Hcit peptides with their wt equivalents demonstrated a higher binding affinity of the Hcit containing peptides than the corresponding wt peptides for both Aldo 209–217 and Cyk8 117–125 suggesting the Hcit modification in these two epitopes improved binding to HLA-A2 (figure 3C,D). It should be noted that the better binding affinity of the shorter peptides might be an artifact of the vitro assay relying on direct binding of peptide to MHC-I rather than any uptake, processing, and presentation. However, these results confirm that MHC-I binding affinity can be improved as a result of the Hcit modification.

Immunization with the short peptides induces high frequency CD8 responses

To confirm the immunogenicity of the shorter peptides, HLA-A2 transgenic (HHDII) mice were immunized with either the shorter Aldo 209-217Hcit or Cyk8 117-125Hcit peptide. IFNγ responses were then evaluated against the Hcit and wt versions of the shorter and longer peptides. Shorter peptides stimulated a high frequency T-cell response specific to the shorter modified peptides that reacted with the longer modified peptides (online supplemental figure 4A). These responses were also seen in HHDII/DR1 mice (online supplemental figure 4B) and reflects that longer peptides are likely to be processed and presented as shorter epitopes to T cells.

To confirm that the shorter peptides induce only CD8-mediated responses, responses were assessed by intracellular cytokine staining for IFNγ (online supplemental figure 4C). Staining profiles clearly showed an IFNγ response in peptide stimulated splenocytes within the CD8 T-cell population and not within the CD4 T cells (Gating strategy in online supplemental figure 5A,B).

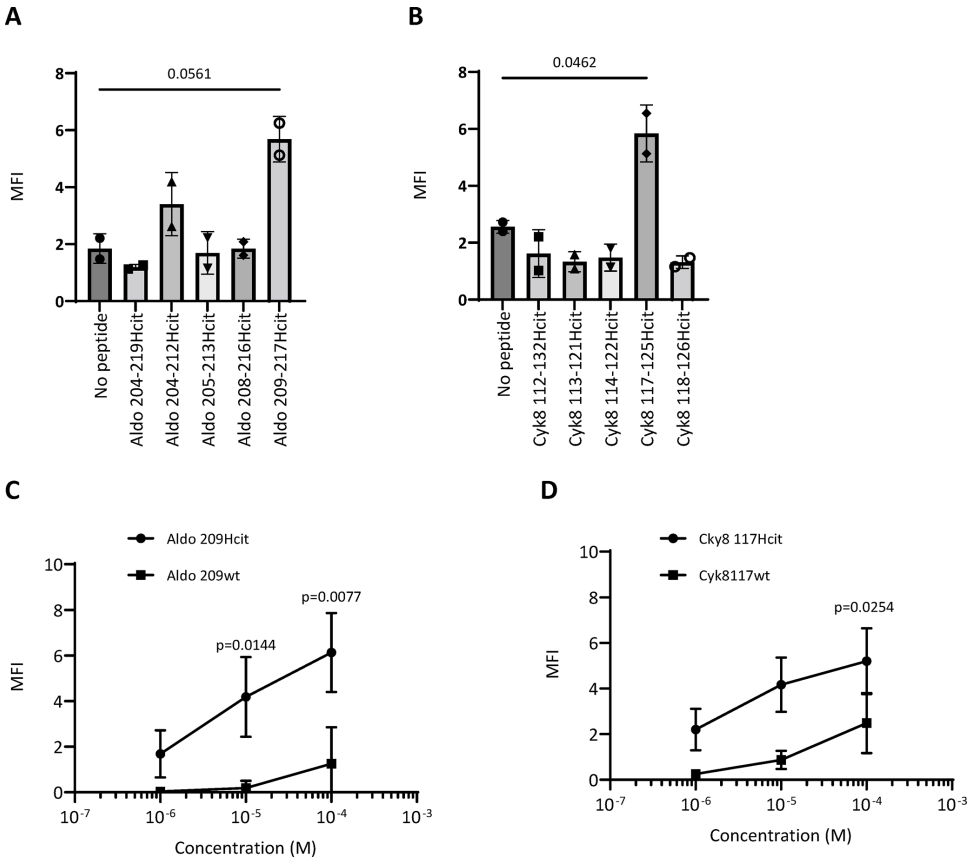

Figure 3 Modified peptides show better binding to MHC class I. Binding affinity of the shorter Aldo and Cyk8 peptides selected based on IEDB predicted scores to HLA-A2 was determined in T2 binding assay (A and B) at 100 µg/mL of peptide concentration. Data from two independent experiments are presented. Paired t-test was performed to derive statistical significance. HLA-A2 binding affinity of Aldo 209-217Hcit or Cyk8 117-125Hcit was compared with the corresponding wt peptides at different peptide concentrations (C and D). Data from three independent experiments are presented and two-way ANOVA has been performed for statistical analysis. Aldo, aldolase A; ANOVA, analysis of variance; Cyk8, cytokeratin 8; Hcit, homocitrulline; HLA, human leukocyte antigen; IEDB, Immune Epitope Database; MFI, median fluorescence intensity; MHC, major histocompatibility complex; wt, wild type.

Splenocytes expanded in vitro also showed co-expression of IFNγ and activation induced costimulatory molecule CD137 in response to peptide and peptide pulsed HHDII expressing cells. Example staining for Aldo 209-217Hcit response is shown in online supplemental figure 6. To assess the memory phenotype of these CD8 responses, splenocytes from mice taken at either day 21 or day 46 post immunization, were stimulated ex vivo with peptide and analyzed for the expression of the memory markers, CD62L and CD127 (IL-7Ra) on IFNγ positive CD8 T cells (online supplemental figure 7). Combined analysis of CD62L and CD127 expression on Aldo 209-217Hcit specific CD8 cells showed that cells were mainly of effector cells (CD62L−CD127−), with a small portion of effector memory (CD62L−CD127+) at day 21 post vaccination. At day 46 following immunization, a higher frequency of central (CD62L+CD127+) and effector memory phenotype was observed.

Further confirmation that responses induced by immunization with the shorter peptides were CD8-mediated was provided using anti-CD4 or anti-CD8 antibody in ELISpot assay. Response to Aldo 209-217Hcit was also efficiently blocked by anti-CD8 antibody in HHDII mice but was unaffected by either antibody in HHDII/DR1 strain (online supplemental figure 8A,B). It appeared that this response was too strong to show blocking at the concentration of the CD8 antibody used in the assay. The presence of CD8 antibody, but not CD4 significantly inhibited Aldo 209-217Hcit response when 100 times less peptide and half the number of cells were used in the assay on cultured HHDII/DR1 splenocytes (online supplemental figure 8C). The use of anti-CD8 blocking antibody clearly blocked the response to Cyk8 117-125Hcit in both HHDII and HHDII/DR1 models, whereas anti-CD4 antibody had no effect (online supplemental figure 8A,B).

Tumors show an increase in homocitrullinated proteins and Aldo209-217Hcit specific CD8 response mediates efficient antitumor therapy

MDSCs present in the tumor microenvironment can be a source of MPO that can drive homocitrullination. Previous studies have demonstrated that MDSCs in the tumor environment in a mouse B16 melanoma model can mediate homocitrullination.[17] We confirmed here

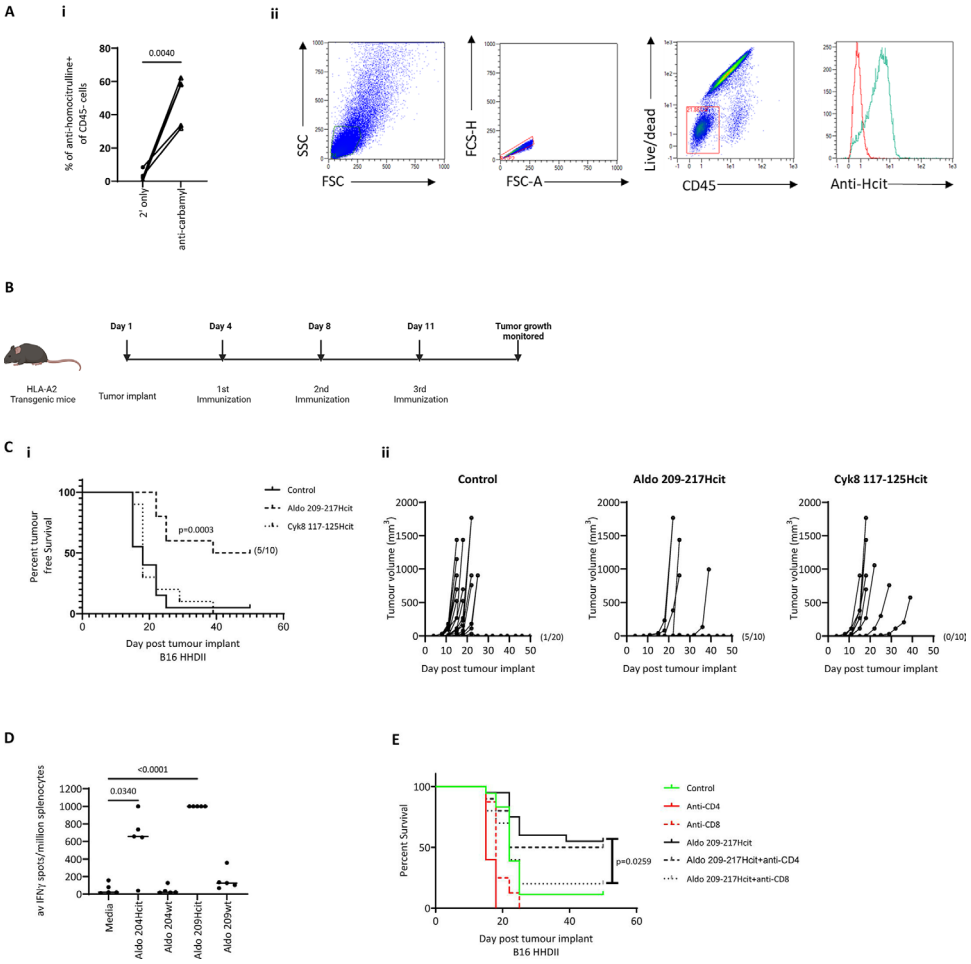

Figure 4 Aldo 209-217Hcit specific immune responses mediate efficient anti-tumor therapy in vivo. Expression of Hcit proteins in in-vivo grown B16 tumor was assessed by staining with anti-Hcit antibody. Paired analysis was performed comparing secondary (2') antibody control to samples stained with anti-Hcit (anti-carbamyl) antibody for multiple tumors (Ai). An example of gating strategy is shown (Aii) where sequential gating was performed. FSC/SSC gated cells were doublet excluded using FSC-A/FCS-H gating. Events were then gated to include live cells negative for CD45 expression. Live/CD45– cells were then assessed for staining with anti-Hcit antibody (green histogram) or the secondary antibody alone (red histogram) (Aii). In vivo antitumor study was performed in HHDII mice implanted with B16HHDII tumor on day 1. Mice were then immunized with Aldo 209-217Hcit or Cyk8 117-125Hcit on day 4, 8 and 11 as shown in the schematic (B). Statistical analysis compares overall survival between unimmunized control mice and peptide immunized mice (Cii). Study is a representative data set for which each group contained n≥10 mice and significant p values are shown (Mantel-Cox test). Tumor growth for individual mice is shown for each group (Cii) and the number of tumor-free mice out of the total number of mice per group used in the experiment is given in brackets. Immune responses in the survivors (n=5) from Aldo 209-217Hcit immunized group was evaluated in IFNγ ELISpot assay (D). Symbols represent mean IFNγ spots/million splenocytes for individual mice and line represents median value between mice. Significant p values are shown (paired ANOVA). HHDII mice implanted with B16 HHDII cells were treated with either anti-CD4 or anti-CD8 antibody with or without Aldo 209-217Hcit immunization (E). The statistical difference in survival between peptide vaccinated mice and mice receiving anti-CD8 depletion antibody in combination with peptide is presented where for all the groups, n≥5 (Mantel-Cox test). Aldo, aldolase A; ANOVA, analysis of variance; Cyk8, cytokeratin 8; FSC, forward scatter light; FSC-A, FSC-Area; FSC-H, FSC-Height; ELISpot, enzyme linked immunosorbent spot; Hcit, homocitrulline; HLA, human leukocyte antigen; IFNγ, interferon γ, SSC, side scatter light.

the presence of homocitrullination within in vivo grown murine B16 tumors. Tumor ex vivo showed staining with the anti-Hcit antibody over that of the secondary only control (figure 4Ai,ii). Thus, suggesting that this tumor model is suitable to study homocitrulline specific immune responses.

To determine if the modified peptides described in this study are naturally processed and presented by tumor cells, and if the specific CD8 responses are of high enough avidity to recognize these, we analyzed whether immunization had a significant effect on tumor growth in vivo. HHDII mice were implanted with B16 HHDII melanoma cells expressing HHDII and murine MHC-II and then immunized with Aldo 209-217Hcit or Cyk8 117-125Hcit peptide (figure 4B, immunization regime). Aldo 209-217Hcit immunized mice showed significantly improved survival and delayed tumor growth when compared with unimmunized control mice (p=0.0003)

(figure 4C, online supplemental figure 9 shows representative tumor images). No effect on survival was seen in mice immunized with Cyk8 117-125Hcit peptide. Analysis of immune responses in the survivors demonstrated Aldo 209-217Hcit specific response in these mice that reacted with the longer Aldo 204-219Hcit peptide but not to the wt peptides (figure 4D). Thus, providing evidence of efficient immune responses even in the presence of tumor. Interestingly, despite inducing a potent CD8 T-cell response, Cyk8 117-125Hcit peptide failed to provide any in vivo antitumor effect (figure 4C). Tumor challenge experiment in HHDII mice also provided significant antitumor effect following immunization with longer Aldo 204-219Hcit or shorter Aldo 209–217wt peptides (online supplemental figure 10A). Immune response analysis from the survivors of Aldo 209–217wt immunized group showed response to both Aldo 204-219Hcit and Aldo 209-217Hcit peptides but not to the immunizing peptide (online supplemental figure 10B), even though in non-tumor bearing mice immunization with the wt peptide does not induce any detectable response (figure 2B).

To prove that CD8 T cells are responsible for the antitumor response induced by Aldo 209-217Hcit peptide immunization, the effect of peptide vaccination was assessed following in vivo depletion of CD8 or CD4 T cells (figure 4E). The efficiency of depletion was confirmed in animals at day 2 post treatment (online supplemental figure 11). Survival in Aldo 209-217Hcit peptide vaccinated mice was reduced significantly with CD8 depletion (p=0.0259), but not by CD4 depletion (p=0.3261), confirming that CD8 T cells mediate the antitumor effect induced by Aldo 209-217Hcit peptide vaccination.

CD8 response avidity or lack of cytotoxicity cannot explain the absence of tumor therapy

The absence of tumor therapy after Cyk8 117-125Hcit peptide immunization may be due to lower avidity responses stimulated by the shorter Cyk8 117-125Hcit peptide or due to differences in cytotoxic ability of the immune responses generated. To investigate this, we assessed the avidity of responses stimulated by the shorter Aldo 209-217Hcit or Cyk8 117-125Hcit peptides. Peptide titration analysis revealed that higher avidity responses to the shorter peptides were detected and there was little difference between the avidity of the Cyk8 117-125Hcit response (EC_{50}=5.412e^{-011}) and the Aldo 209-217Hcit response (EC_{50}=4.692e^{-010}) (figure 5A). This suggests that the lack of tumor therapy was not due to the avidity of the individual responses induced.

Having demonstrated that high avidity Hcit peptide specific CD8 responses can be generated, the ability of these cells to recognize and kill target cells was investigated. Splenocytes from HHDII mice immunized with Aldo 209-217Hcit or Cyk8 117-125Hcit were expanded in vitro in the presence of the immunizing peptide and cultures were tested for killing of T2 cells pulsed with Hcit peptide or wt peptide. Both peptide cultures showed specific lysis of Hcit peptide pulsed T2 cells compared with T2 cells alone or T2 cells pulsed with wt peptide (figure 5Bi,ii). In addition to target cell killing, both responses induced in HHDII/DR1 mice demonstrated significant IFNγ responses to Hcit peptide pulsed T2/DR1 cells (online supplemental figure 12), confirming both cytokine release and cytotoxic responses to Hcit peptide expressing target cells but not to those expressing wt peptides. This data suggests that the potent Hcit specific CD8 T-cell responses are both capable of recognizing and killing cells presenting modified peptide on MHC-I. Therefore, this cannot provide the explanation for the lack of tumor therapy by the Cyk8 117-125Hcit peptide vaccination, and it is likely that the Cyk8 117-125Hcit peptide is not naturally homocitrullinated or presented in the tumor. Cyk8 117-125Hcit peptide contains multiple Hcit residues (positions 117 and 122) and the importance of modification at each of these positions was assessed. HHDII mice immunized with Cyk8 117-125Hcit peptide failed to respond to longer Cyk8 112-132Hcit or shorter Cyk8 117-125Hcit peptide that contains a single Hcit residue either at position 117 or 122 (online supplemental figure 13A). In addition, in vitro T2 binding assays showed that partial homocitrullination reduces the MHC-I binding affinity of Cyk8 117–125 peptide suggesting that single Hcit peptide does not efficiently bind to HLA-A2 and therefore is unlikely to be naturally presented (online supplemental figure 13B). This data suggests that the immune response stimulated requires homocitrullination at both position 117 and 122 but it remains likely that both lysine residues are not homocitrullinated in vivo resulting in lack of tumor recognition. Aldo 209-217Hcit peptide contains a single Hcit residue and Aldo 209-217Hcit specific response reacted with the longer peptide containing Hcit residues at positions 208 and 215 but was reduced to the longer peptide containing only Hcit residue that is within the short epitope (position 215) suggesting although the Hcit at position 208 is not within the short epitope sequence it may be important for cleavage and presentation (online supplemental figure 13C).

Homocitrullinated Aldo209-217 is detected in human tumors

We have demonstrated therapy of murine B16 tumor by Aldo 209-217Hcit specific CD8 responses suggesting presentation of this peptide. We sought to examine lysates of frozen human tumor samples by targeted mass spectrometry to determine if the Aldo 209-217Hcit or Cyk8 117-125Hcit peptides are present in human tumor. Ten independent human tumor samples (three ovarian, three head and neck, two renal and two triple negative breast cancer, online supplemental table 3) were analyzed for presence of the homocitrullinated peptides.

Homocitrullinated Aldo 209-217 peptide was detected in 9 out of 10 tumor samples whereas the longer Aldo 204-219Hcit peptide was not detected in any samples and neither were either the longer or shorter homocitrullinated Cyk8 peptides (table 1). Figure 6 shows representative fragment ion traces for detection of Aldo 209-217Hcit in four

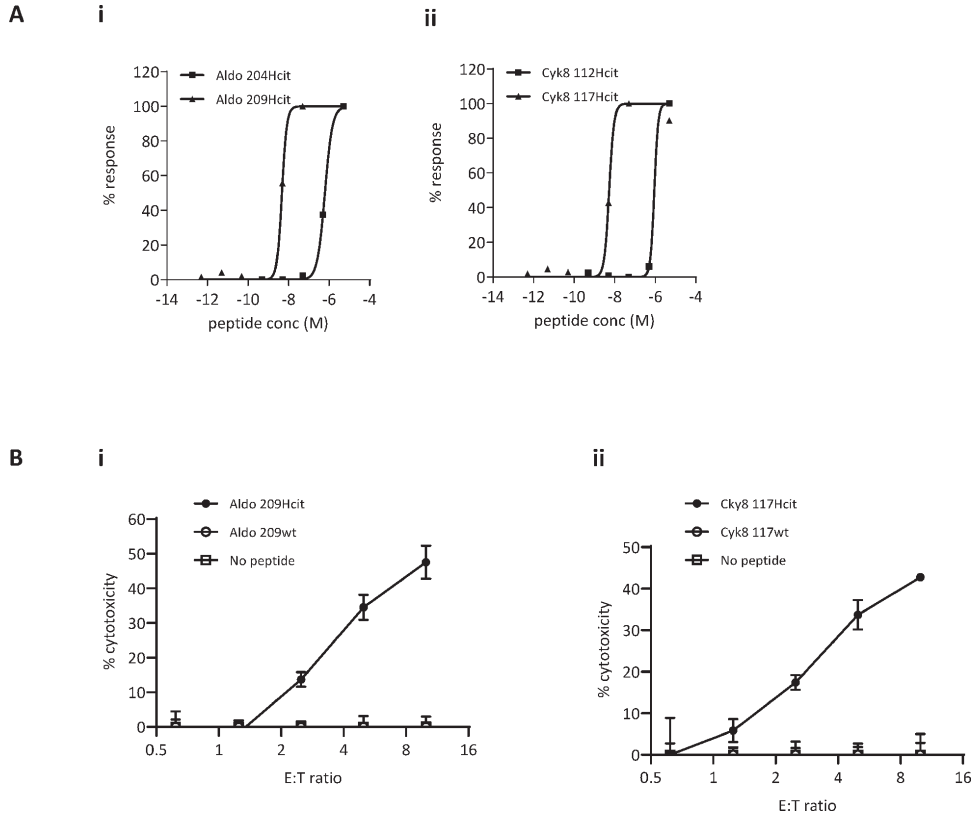

Figure 5 Response avidity and cytotoxicity are not responsible for absence of tumor therapy from Cyk8 117-125Hcit vaccination. HLA-A2 transgenic mice (HHDII) were immunized with either Aldo 209-217Hcit or Cyk8 117-125Hcit peptide and peptide titrations of short and long Hcit peptides were performed in IFNγ ELISpot (A). Data from three independent experiments are shown for each concentration of peptide and normalized avidity graphs are presented. Splenocyte cultures expanded in vitro in the presence of Aldo 209-217Hcit or Cyk8 117-125Hcit were restimulated with T2 cells pulsed with Hcit or wt peptides. The ability to kill T2 cells pulsed with Hcit or wt peptide was tested in a non-radioactive cell killing assay (B). Data are shown where error bars indicate the variations between quadruplicate assay wells. Aldo, aldolase A; Cyk8, cytokeratin 8; ELISpot, enzyme linked immunosorbent spot; E:T, effector:target; Hcit, homocitrulline; HLA, human leukocyte antigen; IFNγ, interferon γ; wt, wild type.

of the tumor samples compared with an isotopically labeled internal control synthetic peptide. Tumor samples 1, 3, and 7 show unambiguous peptide-derived peak patterns, which look very similar to the internal control. Tumor sample 5 shows a very different pattern and the target peptide cannot be identified with high confidence in this tumor. Although a small number of tumor samples have been analyzed it provides evidence for the presence of the Aldo 209-217Hcit peptide within human tumors.

Together this data shows that homocitrullinated peptides are present in tumors and CD8-mediated, homocitrulline-specific immune responses are capable of targeting these to mediate potent antitumor effects in vivo. However, not all modified peptides are good targets for CD8-mediated tumor therapy as not all may be naturally presented in the tumor.

Table 1 Targeted detection of homocitrullinated peptides by mass spectrometry in human tumor lysates

Antigen	Peptide coordinates	Sequence	Human tumor sample									
			1	2	3	4	5	6	7	8	9	10
Aldolase	209–217	VLAAVY-Hcit-AL	✓	✓	✓	✓	X	✓	✓	✓	✓	✓
Aldolase	204–219	YVTE-Hcit-VLAAVY-Hcit-ALSD	X	X	X	X	X	X	X	X	X	X
Cyk8	117–125	Hcit-MLET-Hcit-WSL	X	X	X	X	X	X	X	X	X	X
Cyk8	112–132	LEQQN-Hcit-MLET- Hcit-WSLLQQQ- Hcit-T	X	X	X	X	X	X	X	X	X	X

Cyk8, cytokeratin 8; Hcit, homocitrulline.

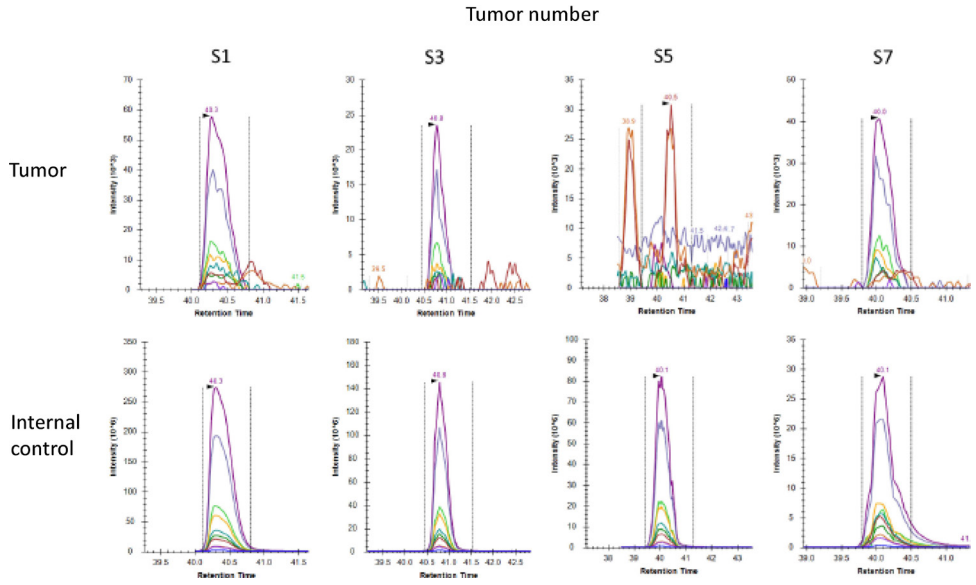

Figure 6 Aldo 209-217Hcit sequence is detected in human tumor samples. Representative fragment ion traces for sequence Aldo 209-217Hcit from four samples. Upper panels show the endogenous tumor channel, lower panel the respective internal standard. Aldo, aldolase A; Hcit, homocitrulline.

DISCUSSION

The success of cancer therapy relies on the stimulation of immune effector functions associated with the generation of tumor antigen specific T-cell responses. There is ample evidence suggesting an important role of CD8 T cells in eradicating tumor cells. Here we present data for a novel class of antigens recognized in the tumor microenvironment by CD8 T cells. Our previous studies have demonstrated that PTM specific CD4 T-cell responses provide survival benefits against aggressive tumor models in mice and suggest the presentation of modified epitopes in the tumor environment.[8–10 12 13 17] In this study, we have shown the first evidence of CD8-mediated responses to Hcit peptides and demonstrated that these are capable of selectively recognizing the modified but not the wt epitopes. CD8 T cells have been shown to specifically recognize epitopes containing other PTMs such as phosphorylation, glycosylation and citrullination.[33–36] Induction of CD8 responses to MHC-I-associated phosphopeptides can target cancer cells[37 38] and tumor-associated MHC-I restricted phosphopeptides have been identified as targets for tumor resident CD8 T cells in colorectal cancer.[39]

A vaccine comprising of Hcit peptides specifically targets modified self-antigens and induces high frequency and high avidity CD8 responses with minimal cross-reactivity to wt peptides. Responses are CD8 T-cell mediated as blockade of CD8 T cells abrogate the responses in vitro and in vivo. High frequency and high avidity Hcit peptide specific CD8 T cells responses generated by vaccination exhibit cytotoxic ability and for the Aldo 209-217Hcit response correlated with efficient antitumor therapy in vivo providing at least 50% survival in the aggressive mouse B16 melanoma model. Thus, providing evidence that homocitrullinated aldolase can be a target for tumor therapy. Despite being capable of inducing high avidity cytotoxic CD8 T cells, vaccination with Cyk8 117-125Hcit peptide failed to provide in vivo tumor protection. Previously we have identified CD4 responses to Hcit peptides from Cyk8 that are expressed in cancers and could be targeted for tumor therapy,[12 17] so the lack of response is not due to low expression of Cyk8. This implies that either the required lysine residue is not converted to Hcit in vivo or the corresponding peptide is not naturally presented on MHC-I by B16 tumors. Indeed, in a small number of human tumors we failed to detect the Cyk8 117-125Hcit peptide suggesting it may not be naturally present. However, we successfully detected the Aldo209-217Hcit peptide implying this could also be an immunotherapy target in human tumors. The Aldo204-219Hcit peptide was not identified suggesting it may be cleaved or is only present as a single Hcit peptide in human tumors. Further analysis would be interesting to determine this and also assess if the native (wt) forms of the peptides can also be detected in human tumors.

High avidity CTLs are effective in antitumor immunity as they require lower antigen concentration for activation and effector cell function.[40 41] However, they are more prone to functional impairment in the immunosuppressive tumor microenvironment.[42 43] CD8 responses are particularly sensitive to inhibitory signals in the tumor environment and antibodies targeting inhibitory receptors on T cells have been used successfully to reduce this inhibition and improve T cell effector function leading to enhanced efficacy of antitumor therapy.[44] Our works and that of others have shown that Programmed cell death protein 1 (PD-1) and Cytotoxic T-lymohocyte-associated protein 4 (CTLA-4) blockade results in the enhancement of the activity of high avidity CD8 T cells induced by vaccination in a preclinical model

and clinical trials of melanoma.[26 31 45–47] Thus, vaccines that can induce strong antitumor CD8 responses will likely be complemented by such checkpoint blockade that are now standard of care in many clinical settings. Despite the presence of these inhibitory mechanisms in the murine B16 melanoma model, the CD8 response stimulated by Aldo 209-217Hcit vaccination in this study provided efficient tumor therapy without the combination of any checkpoint blockade therapy.

This study provides the first evidence of CD8 T-cell responses to homocitrullinated peptides. However, responses to homocitrullinated proteins have been documented in autoimmune diseases and homocitrullinated peptides are known to be presented on MHC-II. Previously we have identified CD4 responses to Hcit peptides from five abundant cytoplasmic proteins including aldolase and Cyk8 that are expressed in cancers and result in antitumor responses.[12 17] Here, we add to this with characterization of Hcit epitopes from aldolase and Cyk8 that stimulate peptide-specific CD8 T-cell responses. The two peptides (Cyk8 117-125Hcit and Aldo 209-217Hcit) identified showed high binding affinity to HLA-A2, a property that is important for the immunogenicity of peptide epitopes. During immune development, T lymphocytes that react strongly to commonly expressed self-antigens are often deleted in the thymus, which is dependent on a number of factors including peptide MHC binding affinity. It is unclear if homocitrullination occurs in the thymus but despite the high peptide binding affinity, the CD8 T-cell repertoires to these two modified self-peptides were not deleted in the murine models tested in this study. In this study, we also demonstrated no evidence of cross reactivity of CD8 responses with the native sequences and there was no evidence of autoimmune effects in the transgenic mouse models used. Indeed, there is little evidence for CD8 T-cell responses in driving autoimmune disease. However, potential autoimmune effects should be closely monitored in any clinical study inducing PTM-specific CD8 responses. Future studies to assess the tumor specific expression of Hcit peptides by performing mass spectrometric analysis of major human organ samples and normal tissue may help elucidate any potential toxicity issues.

This study presents the first evidence that homocitrullinated proteins in the tumor environment can be targeted by vaccination induced CD8 responses to mediate a potent antitumor effect. This may open exciting new avenues for the treatment of cancer using CD8 as well as CD4 responses targeting PTM antigens.

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